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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 48/00, C07H 21/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 96/40266</b> <b>(43) International Publication Date:</b> 19 December 1996 (19.12.96)
<b>(21) International Application Number:</b> PCT/US96/08325 <b>(22) International Filing Date:</b> 3 June 1996 (03.06.96) <b>(30) Priority Data:</b> 08/472,527 7 June 1995 (07.06.95) US <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 08/472,527 (CON) Filed on 7 June 1995 (07.06.95) <b>(71) Applicant (for all designated States except US):</b> EAST CAROLINA UNIVERSITY [US/US]; 210 Spilman Building, Greenville, NC 27858-4353 (US). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> NYCE, Jonathan, W. [US/US]; 903-11 Treybrooke Circle, Greenville, NC 27834 (US). <b>(74) Agents:</b> SIBLEY, Kenneth, D. et al.; Bell, Seltzer, Park & Gibson, P.O. Box Drawer 34009, Charlotte, NC 28234 (US).		<b>(81) Designated States:</b> AL, AM, AT, AT (Utility model), AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> METHOD OF TREATMENT FOR ASTHMA  <b>(57) Abstract</b>  A method of reducing bronchoconstriction in a subject in need of such treatment is disclosed. The method comprises administering to the subject an antisense oligonucleotide molecule directed against the A <sub>1</sub> or A <sub>3</sub> adenosine receptor in an amount effective to reduce bronchoconstriction. The method is useful for treating patients afflicted with asthma. Pharmaceutical formulations are also disclosed.		

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## METHOD OF TREATMENT FOR ASTHMA

This invention was made with Government support under grant RO1CA47217-06 from the National Cancer Institute. The Government has certain rights to this invention.

### 5                   Field of the Invention

This application concerns a method of administering antisense oligonucleotides against the A<sub>1</sub> and A<sub>2</sub> Adenosine receptors as a treatment for asthma.

### Background of the Invention

10                   Asthma is one of the most common diseases in industrialized countries, and in the United States accounts for about 1% of all health care costs. K. Weiss et al., *New Engl. J. Med.* 326, 862-866 (1992). There has been reported an alarming increase in both the prevalence  
15                   and mortality of asthma over the past decade, Asthma-United States, 1980-1990, *MMWR* 41, 733-735 (1992), and occupational asthma is predicted to be the preeminent occupational lung disease in the next decade. M. Chan-Yeung and J. Malo, *European Resp. J.* 7, 346-371 (1994)  
20                   While the increasing mortality of asthma in industrialized countries could be attributable to the increased reliance upon beta agonists in the treatment of this disease, the underlying causes of asthma remain poorly understood. J. Gern and R. Lemanske, In  
25                   *Immunology and Allergy Clinics of North America* 13, Bush, R.K. ed. W.B. Saunders Company, London, pp. 839-860 (1993).

Adenosine may constitute an important natural mediator of bronchial asthma. R. Pauwels et al., *Clinical & Exp. Allergy* 21 Suppl. 1, 48-55 (1991); S. Holgate et al., *Annals of the New York Acad. Sci.* 629, 227-236 (1991). The potential role of adenosine in human asthma is supported by the experimental finding that, in contrast to normal individuals, asthmatic individuals respond to aerosolized adenosine with marked bronchoconstriction. M. Church and S. Holgate, *Trends Pharmacol. Sci.* 7, 49-50 (1986); M. Cushley et al., *Br. J. Clin. Pharmacol.* 15, 161-165 (1983). Similarly, asthmatic rabbits produced using the dust mite allergic rabbit model of human asthma also were shown to respond to aerosolized adenosine with marked bronchoconstriction, while non asthmatic rabbits showed no response. S. Ali et al., *Agents Actions* 37, 165-176 (1992). Recent work using this model system has suggested that adenosine-mediated bronchoconstriction and bronchial hyperresponsiveness in asthma are mediated primarily through the stimulation of adenosine receptors. S. Ali et al., *J. Pharmacol. Exp. Ther.* 268, 1328-1334 (1994); S. Ali et al., *Am. J. Physiol* 266, L271-277 (1994).

Theophylline, an important drug in the treatment of asthma, is a known adenosine receptor antagonist (see M. Cushley et al., *Am. Rev. Resp. Dis.* 129, 380-384 (1984)) and was found to eliminate adenosine-mediated bronchoconstriction in asthmatic rabbits (Ali, et al., *supra*). Pretreatment of allergic rabbits with another A<sub>1</sub>-specific receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), potentially inhibited adenosine-mediated bronchoconstriction and bronchial hyperresponsiveness in allergic rabbits. *Id.* The therapeutic potential, however, of currently available adenosine A<sub>1</sub> receptor-specific antagonists is limited by their toxicity. H. Klitgaard et al., *European J. Pharmacol.* 242, 221-228 (1993). Theophylline has been widely used in the treatment of asthma, but is associated

with frequent, significant toxicity resulting from its narrow therapeutic dose range. E. Powell et al., *Pediatric Emergency Care* 9, 129-133 (1993); S. Nasser and P. Rees, *Drug Safety* 8, 12-18 (1993); P. Epstein, *Annals of Internal Med.* 119, 1216-1217 (1993). The availability of an alternative strategy to downregulate adenosine-mediated bronchoconstriction would clearly be of therapeutic interest.

### Summary of the Invention

10 A first aspect of the present invention is a method of reducing adenosine-mediated bronchoconstriction in a subject in need of such treatment. The method comprises administering an adenosine receptor antisense oligonucleotide to the lungs of the subject in an amount  
15 effective to reduce bronchoconstriction, where the adenosine receptor is selected from the group consisting of A<sub>1</sub> adenosine receptors and A<sub>2</sub> adenosine receptors.

A second aspect of the present invention is a method of treating asthma in a subject in need of such  
20 treatment. The method comprises administering an adenosine receptor antisense oligonucleotide to the lungs of the subject in an amount effective to treat asthma, where the adenosine receptor is selected from the group consisting of A<sub>1</sub> adenosine receptors and A<sub>2</sub> adenosine  
25 receptors.

A third aspect of the present invention is a pharmaceutical composition, comprising, together in a pharmaceutically acceptable carrier, an adenosine receptor antisense oligonucleotide in which the adenosine  
30 receptor is selected from the group consisting of A<sub>1</sub> adenosine receptors and A<sub>2</sub> adenosine receptors, in an amount effective to reduce adenosine-mediated bronchoconstriction.

A fourth aspect of the present invention is the  
35 use of an adenosine receptor antisense oligonucleotide as given above for the preparation of a medicament for (a)

reducing adenosine-mediated bronchoconstriction in a subject in need of such treatment, or (b) treating asthma in a subject in need of such treatment.

Antisense oligonucleotides have received  
5 considerable theoretical consideration as potentially useful pharmacologic agents in human disease. R. Wagner, *Nature* 372, 333-335 (1994). However, practical applications of these molecules in actual models of human disease have been elusive. One important consideration  
10 in the pharmacologic application of these molecules is route of administration. Most experiments utilizing antisense oligonucleotides *in vivo* have involved direct application to limited regions of the brain (see C. Wahlestedt, *Trends in Pharmacological Sciences* 15, 42-46  
15 (1994); J. Lai et al., *Neuroreport* 5, 1049-1052 (1994); K. Standifer et al., *Neuron* 12, 805-810 (1994); A. Akabayashi et al., *Brain Research* 21, 55-61 (1994)), or to spinal fluid (see e.g. L. Tseng et al., *European J. Pharmacol.* 258, R1-3 (1994); R. Raffa et al., *European J. Pharmacol.* 258, R5-7 (1994); F. Gillardon et al.,  
20 *European J. Neurosci.* 6, 880-884 (1994)). Such applications have limited clinical utility due to their invasive nature.

The systemic administration of antisense  
25 oligonucleotides also poses significant problems with respect to pharmacologic application, not the least of which is the difficulty in targeting disease-involved tissues. In contrast, the lung is an excellent potential target for antisense oligonucleotide application since it  
30 may be approached noninvasively and in a tissue-specific manner.

### Brief Description of the Drawings

Figure 1 illustrates the effects of A<sub>1</sub> adenosine receptor antisense oligonucleotides and mismatch control  
35 antisense oligonucleotides on the dynamic compliance of the bronchial airway in a rabbit model. The two stars

represent significant difference at  $p < 0.01$ , Student's t-test.

Figure 2 illustrates the specificity of  $A_1$  adenosine receptor antisense oligonucleotides as indicated by the  $A_1$  and  $A_2$  adenosine receptor number present in airway tissue treated with  $A_1$  adenosine receptor antisense oligonucleotides.

### Detailed Description of the Invention

Nucleotide sequences are presented herein by single strand only, in the 5' to 3' direction, from left to right. Nucleotides and amino acids are represented herein in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission, or (for amino acids) by three letter code, in accordance with 37 CFR §1.822 and established usage. See, e.g., Patent In User Manual, 99-102 (Nov. 1990) (U.S. Patent and Trademark Office, Office of the Assistant Commissioner for Patents, Washington, D.C. 20231); U.S. Patent No. 4,871,670 to Hudson et al. at Col. 3 lines 20-43 (applicants specifically intend that the disclosure of this and all other patent references cited herein be incorporated herein by reference).

The method of the present invention may be used to reduce adenosine-mediated bronchoconstriction in the lungs of a subject for any reason, including (but not limited to) asthma. Antisense oligonucleotides to the  $A_1$  and  $A_2$  receptors are shown to be effective in the downregulation of  $A_1$  or  $A_2$  in the cell. One novel feature of this treatment, as compared to traditional treatments for adenosine-mediated bronchoconstriction, is that administration is direct to the lungs. Additionally, a receptor protein itself is reduced in amount, rather than merely interacting with a drug, and toxicity is reduced.

As used herein, the term "treat" or "treating" asthma refers to a treatment which decreases the likelihood that the subject administered such treatment

-6-

will manifest symptoms of bronchoconstriction or asthma. The term "downregulate" refers to inducing a decrease in production, secretion or availability (and thus a decrease in concentration) of intracellular A<sub>1</sub> or A<sub>2</sub> adenosine receptor.

The present invention is concerned primarily with the treatment of human subjects but may also be employed for the treatment of other mammalian subjects, such as dogs and cats, for veterinary purposes.

In general, "antisense" refers to the use of small, synthetic oligonucleotides, resembling single-stranded DNA, to inhibit gene expression by inhibiting the function of the target messenger RNA (mRNA). Milligan, J.F. et al., *J. Med. Chem.* 36(14), 1923-1937 (1993). In the present invention, inhibition of gene expression of the A<sub>1</sub> or A<sub>2</sub> adenosine receptor is desired. Gene expression is inhibited through hybridization to coding (sense) sequences in a specific messenger RNA (mRNA) target by hydrogen bonding according to Watson-Crick base pairing rules. The mechanism of antisense inhibition is that the exogenously applied oligonucleotides decrease the mRNA and protein levels of the target gene or cause changes in the growth characteristics or shapes of the cells. *Id.* See also Helene, C. and Toulme, J., *Biochim. Biophys. Acta* 1049, 99-125 (1990); Cohen, J.S., Ed., *Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression*; CRC Press: Boca Raton, FL (1987).

As used herein, "adenosine receptor antisense oligonucleotide" is defined as a short sequence of synthetic nucleotides that (1) hybridizes to any coding sequence in an mRNA which codes for the A<sub>1</sub> adenosine receptor or A<sub>2</sub> adenosine receptor, according to hybridization conditions described below, and (2) upon hybridization causes a decrease in gene expression of the A<sub>1</sub> or A<sub>2</sub> adenosine receptor.



The mRNA sequence of the A<sub>1</sub> or A<sub>2</sub> adenosine receptor is derived from the DNA base sequence of the gene expressing either the A<sub>1</sub> or A<sub>2</sub> adenosine receptor. The sequence of the genomic human A<sub>1</sub> adenosine receptor is known and is disclosed in U.S. Patent No. 5,320,963 to G. Stiles et al. The A<sub>2</sub> adenosine receptor has been cloned, sequenced and expressed in rat (see F. Zhou et al., *Proc. Nat'l Acad. Sci. USA* 89:7432 (1992)) and human (see M.A. Jacobson et al., U.K. Patent Application No. 9304582.1 (1993)). Thus, antisense oligonucleotides that downregulate the production of the A<sub>1</sub> or A<sub>2</sub> adenosine receptor may be produced in accordance with standard techniques.

One aspect of this invention is an antisense oligonucleotide having a sequence capable of binding specifically with any sequence of an mRNA molecule which encodes a human A<sub>1</sub> adenosine receptor or A<sub>2</sub>-adenosine receptor so as to prevent translation of the mRNA molecule. The antisense oligonucleotide may have a sequence disclosed herein in SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

Chemical analogs of oligonucleotides (e.g., oligonucleotides in which the phosphodiester bonds have been modified, e.g., to the methylphosphonate, the phosphotriester, the phosphorothioate, the phosphorodithioate, or the phosphoramidate, so as to render the oligonucleotide more stable in vivo) are also an aspect of the present invention. The naturally occurring phosphodiester linkages in oligonucleotides are susceptible to degradation by endogenously occurring cellular nucleases, while many analogous linkages are highly resistant to nuclease degradation. See Milligan et al., and Cohen, J.S., *supra*. Protection from degradation can be achieved by use of a "3'-end cap" strategy by which nuclease-resistant linkages are substituted for phosphodiester linkages at the 3' end of the oligonucleotide. See Tidd, D.M. and Warenus, H.M.,

Br. J. Cancer 60, 343-350 (1989); Shaw, J.P. et al.,  
Nucleic Acids Res. 19, 747-750 (1991). Phosphoramidates,  
phosphorothioates, and methylphosphonate linkages all  
function adequately in this manner. More extensive  
5 modification of the phosphodiester backbone has been  
shown to impart stability and may allow for enhanced  
affinity and increased cellular permeation of  
oligonucleotides. See Milligan, et al., supra. Many  
different chemical strategies have been employed to  
10 replace the entire phosphodiester backbone with novel  
linkages. *Id.* Backbone analogues include  
phosphorothioate, phosphorodithioate, methylphosphonate,  
phosphoramidate, boranophosphate, phosphotriester,  
formacetal, 3'-thioformacetal, 5'-thioformacetal, 5'-  
15 thioether, carbonate, 5'-N-carbamate, sulfate, sulfonate,  
sulfamate, sulfonamide, sulfone, sulfite, sulfoxide,  
sulfide, hydroxylamine, methylene(methylimino) (MMI) or  
methyleneoxy(methylimino) (MOMI) linkages.  
Phosphorothioate and methylphosphonate-modified  
20 oligonucleotides are particularly preferred due to their  
availability through automated oligonucleotide synthesis.  
*Id.* Where appropriate, the antisense oligonucleotides  
may be administered in the form of their pharmaceutically  
acceptable salts.

25 Antisense oligonucleotides may be of any  
suitable length (e.g., from about 10 to 60 nucleotides in  
length), depending on the particular target being bound  
and the mode of delivery thereof. Preferably the  
antisense oligonucleotide is directed to an mRNA region  
30 containing a junction between intron and exon. Where the  
antisense oligonucleotide is directed to an intron/exon  
junction, it may either entirely overlie the junction or  
may be sufficiently close to the junction to inhibit  
splicing out of the intervening exon during processing of  
35 precursor mRNA to mature mRNA (e.g., with the 3' or 5'  
terminus of the antisense oligonucleotide being is  
positioned within about, for example, 10, 5, 3, or 2

nucleotides of the intron/exon junction). Also preferred are antisense oligonucleotides which overlap the initiation codon.

When practicing the present invention, the  
5 antisense oligonucleotides administered may be related in origin to the species to which it is administered. When treating humans, human antisense may be used if desired.

Pharmaceutical compositions comprising an antisense oligonucleotide as given above effective to  
10 reduce expression of an  $A_1$  or  $A_3$  adenosine receptor by passing through a cell membrane and binding specifically with mRNA encoding an  $A_1$  or  $A_3$  adenosine receptor in the cell so as to prevent its translation are another aspect of the present invention. Such compositions are provided  
15 in a suitable pharmaceutically acceptable carrier (e.g., sterile pyrogen-free saline solution). The antisense oligonucleotides may be formulated with a hydrophobic carrier capable of passing through a cell membrane (e.g., in a liposome, with the liposomes carried in a  
20 pharmaceutically acceptable aqueous carrier). The oligonucleotides may also be coupled to a substance which inactivates mRNA, such as a ribozyme. Such oligonucleotides may be administered to a subject to inhibit the activation of  $A_1$  or  $A_3$  adenosine receptors,  
25 which subject is in need of such treatment for any of the reasons discussed herein. Furthermore, the pharmaceutical formulation may also contain chimeric molecules comprising antisense oligonucleotides attached to molecules which are known to be internalized by cells.  
30 These oligonucleotide conjugates utilize cellular uptake pathways to increase cellular concentrations of oligonucleotides. Examples of macromolecules used in this manner include transferrin, asialoglycoprotein (bound to oligonucleotides via polylysine) and  
35 streptavidin.

In the pharmaceutical formulation the antisense compound may be contained within a lipid particle or

-10-

vesicle, such as a liposome or microcrystal. The particles may be of any suitable structure, such as unilamellar or plurilamellar, so long as the antisense oligonucleotide is contained therein. Positively charged  
5 lipids such as N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl-ammoniummethylsulfate, or "DOTAP," are particularly preferred for such particles and vesicles.

The preparation of such lipid particles is well known. See, e.g., U.S. Patent Nos. 4,880,635 to Janoff et al.;  
10 4,906,477 to Kurono et al.; 4,911,928 to Wallach; 4,917,951 to Wallach; 4,920,016 to Allen et al.; 4,921,757 to Wheatley et al.; etc.

Subjects may be administered the active composition by any means which transports the antisense  
15 nucleotide composition to the lung. The antisense compounds disclosed herein may be administered to the lungs of a patient by any suitable means, but are preferably administered by generating an aerosol comprised of respirable particles, the respirable  
20 particles comprised of the antisense compound, which particles the subject inhales. The respirable particles may be liquid or solid. The particles may optionally contain other therapeutic ingredients.

Particles comprised of antisense compound for  
25 practicing the present invention should include particles of respirable size: that is, particles of a size sufficiently small to pass through the mouth and larynx upon inhalation and into the bronchi and alveoli of the lungs. In general, particles ranging from about .5 to 10  
30 microns in size are respirable. Particles of non-respirable size which are included in the aerosol tend to deposit in the throat and be swallowed, and the quantity of non-respirable particles in the aerosol is preferably minimized. For nasal administration, a particle size in  
35 the range of 10-500  $\mu\text{m}$  is preferred to ensure retention in the nasal cavity.

Liquid pharmaceutical compositions of active compound for producing an aerosol can be prepared by combining the antisense compound with a suitable vehicle, such as sterile pyrogen free water. Other therapeutic compounds may optionally be included.

Solid particulate compositions containing respirable dry particles of micronized antisense compound may be prepared by grinding dry antisense compound with a mortar and pestle, and then passing the micronized composition through a 400 mesh screen to break up or separate out large agglomerates. A solid particulate composition comprised of the antisense compound may optionally contain a dispersant which serves to facilitate the formation of an aerosol. A suitable dispersant is lactose, which may be blended with the antisense compound in any suitable ratio (e.g., a 1 to 1 ratio by weight). Again, other therapeutic compounds may also be included.

The dosage of the antisense compound administered will depend upon the disease being treated, the condition of the subject, the particular formulation, the route of administration, the timing of administration to a subject, etc. In general, intracellular concentrations of the oligonucleotide of from .05 to 50  $\mu\text{M}$ , or more particularly .2 to 5  $\mu\text{M}$ , are desired. For administration to a subject such as a human, a dosage of from about .01, .1, or 1 mg/Kg up to 50, 100, or 150 mg/Kg or more is typically employed. Depending on the solubility of the particular formulation of active compound administered, the daily dose may be divided among one or several unit dose administrations. Administration of the antisense compounds may be carried out therapeutically (i.e., as a rescue treatment) or prophylactically.

Aerosols of liquid particles comprising the antisense compound may be produced by any suitable means, such as with a nebulizer. See, e.g., U.S. Patent No.

-12-

4,501,729. Nebulizers are commercially available devices which transform solutions or suspensions of the active ingredient into a therapeutic aerosol mist either by means of acceleration of a compressed gas, typically air or oxygen, through a narrow venturi orifice or by means of ultrasonic agitation. Suitable formulations for use in nebulizers consist of the active ingredient in a liquid carrier, the active ingredient comprising up to 40% w/w of the formulation, but preferably less than 20% w/w. the carrier is typically water or a dilute aqueous alcoholic solution, preferably made isotonic with body fluids by the addition of, for example, sodium chloride. Optional additives include preservatives if the formulation is not prepared sterile, for example, methyl hydroxybenzoate, antioxidants, flavoring agents, volatile oils, buffering agents and surfactants.

Aerosols of solid particles comprising the active compound may likewise be produced with any solid particulate medicament aerosol generator. Aerosol generators for administering solid particulate medicaments to a subject produce particles which are respirable, as explained above, and generate a volume of aerosol containing a predetermined metered dose of a medicament at a rate suitable for human administration. One illustrative type of solid particulate aerosol generator is an insufflator. Suitable formulations for administration by insufflation include finely comminuted powders which may be delivered by means of an insufflator or taken into the nasal cavity in the manner of a snuff. In the insufflator, the powder (e.g., a metered dose thereof effective to carry out the treatments described herein) is contained in capsules or cartridges, typically made of gelatin or plastic, which are either pierced or opened *in situ* and the powder delivered by air drawn through the device upon inhalation or by means of a manually-operated pump. The powder employed in the insufflator consists either solely of the active

ingredient or of a powder blend comprising the active ingredient, a suitable powder diluent, such as lactose, and an optional surfactant. The active ingredient typically comprises from 0.1 to 100 w/w of the formulation. A second type of illustrative aerosol generator comprises a metered dose inhaler. Metered dose inhalers are pressurized aerosol dispensers, typically containing a suspension or solution formulation of the active ingredient in a liquified propellant. During use these devices discharge the formulation through a valve adapted to deliver a metered volume, typically from 10 to 150  $\mu$ l, to produce a fine particle spray containing the active ingredient. Suitable propellants include certain chlorofluorocarbon compounds, for example, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane and mixtures thereof. The formulation may additionally contain one or more co-solvents, for example, ethanol, surfactants, such as oleic acid or sorbitan trioleate, antioxidants and suitable flavoring agents.

The aerosol, whether formed from solid or liquid particles, may be produced by the aerosol generator at a rate of from about 10 to 150 liters per minute, more preferably from about 30 to 150 liters per minute, and most preferably about 60 liters per minute. Aerosols containing greater amounts of medicament may be administered more rapidly.

The following examples are provided to illustrate the present invention, and should not be construed as limiting thereon. In these examples,  $\mu$ M means micromolar, mL means milliliters,  $\mu$ m means micrometers, mm means millimeters, cm means centimeters,  $^{\circ}$ C means degrees Celsius,  $\mu$ g means micrograms, mg means milligrams, g means grams, kg means kilograms, M means molar, and h means hours.

**EXAMPLE 1**Design and synthesis of antisense oligonucleotides

The design of antisense oligonucleotides against the A<sub>1</sub> and A<sub>2</sub> adenosine receptors may require the solution of the complex secondary structure of the target A<sub>1</sub> receptor mRNA and the target A<sub>2</sub> receptor mRNA. After generating this structure, antisense nucleotides are designed which target regions of mRNA which might be construed to confer functional activity or stability to the mRNA and which optimally may overlap the initiation codon. Other target sites are readily usable. As a demonstration of specificity of the antisense effect, other oligonucleotides not totally complementary to the target mRNA, but containing identical nucleotide compositions on a w/w basis, are included as controls in antisense experiments.

Adenosine A<sub>1</sub> receptor mRNA secondary structure was analyzed and used as described above to design a phosphorothioate antisense oligonucleotide. The antisense oligonucleotide which was synthesized was designated **HAdA1AS** and had the following sequence:

5'-GAT GGA GGG CGG CAT GGC GGG-3' (SEQ ID NO:1)

As a control, a mismatched phosphorothioate antisense nucleotide designated **HAdA1MM** was synthesized with the following sequence:

5'-GTA GCA GGC GGG GAT GGC GGC-3' (SEQ ID NO:2)

Each oligonucleotide had identical base content and general sequence structure. Homology searches in GENBANK (release 85.0) and EMBL (release 40.0) indicated that the antisense oligonucleotide was specific for the human and rabbit adenosine A<sub>1</sub> receptor genes, and that the mismatched control was not a candidate for hybridization with any known gene sequence.



-15-

Adenosine A<sub>1</sub> receptor mRNA secondary structure was similarly analyzed and used as described above to design two phosphorothioate antisense oligonucleotides. The first antisense oligonucleotide (HAdA3AS1) synthesized had the following sequence:

5'-GTT GTT GGG CAT CTT GCC-3' (SEQ ID NO:3)

As a control, a mismatched phosphorothioate antisense oligonucleotide (HAdA3MM1) was synthesized, having the following sequence:

10 5'-GTA CTT GCG GAT CTA GGC-3' (SEQ ID NO:4)

A second phosphorothioate antisense oligonucleotide (HAdA3AS2) was also designed and synthesized, having the following sequence:

5'-GTG GGC CTA GCT CTC GCC-3' (SEQ ID NO:5)

15 Its control oligonucleotide (HAdA3MM2) had the sequence:

5'-GTC GGG GTA CCT GTC GGC-3' (SEQ ID NO:6)

Phosphorothioate oligonucleotides were synthesized on an Applied Biosystems Model 396 Oligonucleotide Synthesizer, and purified using NENSORB chromatography (DuPont, MD).

## EXAMPLE 2

### Testing of A<sub>1</sub>-Adenosine Receptor

#### Antisense Oligonucleotides in vitro

The antisense oligonucleotide against the human A<sub>1</sub> receptor (SEQ ID NO:1) described above was tested for efficacy in an in vitro model utilizing lung adenocarcinoma cells HTB-54. HTB-54 lung adenocarcinoma cells were demonstrated to express the A<sub>1</sub> adenosine receptor using standard northern blotting procedures and

-16-

receptor probes designed and synthesized in the laboratory.

HTB-54 human lung adenocarcinoma cells (106/100 mm tissue culture dish) were exposed to 5.0  $\mu$ M **HAdA1AS** or **HAdA1MM** for 24 hours, with a fresh change of media and oligonucleotides after 12 hours of incubation. Following 24 hour exposure to the oligonucleotides, cells were harvested and their RNA extracted by standard procedures. A 21-mer probe corresponding to the region of mRNA targeted by the antisense (and therefore having the same sequence as the antisense, but not phosphorothioated) was synthesized and used to probe northern blots of RNA prepared from **HAdA1AS**-treated, **HAdA1MM**-treated and non-treated HTB-54 cells. These blots showed clearly that **HAdA1AS** but not **HAdA1MM** effectively reduced human adenosine receptor mRNA by >50%. This result showed that **HAdA1AS** is a good candidate for an anti-asthma drug since it depletes intracellular mRNA for the adenosine A<sub>1</sub> receptor, which is involved in asthma.

20

### EXAMPLE 3

#### Efficacy of A<sub>1</sub>-Adenosine Receptor

#### Antisense Oligonucleotides in vivo

A fortuitous homology between the rabbit and human DNA sequences within the adenosine A<sub>1</sub> gene overlapping the initiation codon permitted the use of the phosphorothioate antisense oligonucleotides initially designed for use against the human adenosine A<sub>1</sub> receptor in a rabbit model.

Neonatal New Zealand white Pasteurella-free rabbits were immunized intraperitoneally within 24 hours of birth with 312 antigen units/mL house dustmite (*D. farinae*) extract (Berkeley Biologicals, Berkeley, CA), mixed with 10% kaolin. Immunizations were repeated weekly for the first month and then biweekly for the next 2 months. At 3-4 months of age, eight sensitized rabbits were anesthetized and relaxed with a mixture of ketamine

-17-

hydrochloride (44 mg/kg) and acepromazine maleate (0.4 mg/kg) administered intramuscularly.

The rabbits were then laid supine in a comfortable position on a small molded, padded animal board and intubated with a 4.0-mm intratracheal tube (Mallinkrodt, Inc., Glens Falls, NY). A polyethylene catheter of external diameter 2.4 mm with an attached latex balloon was passed into the esophagus and maintained at the same distance (approximately 16 cm) from the mouth throughout the experiments. The intratracheal tube was attached to a heated Fleisch pneumotachograph (size 00; DOM Medical, Richmond, VA), and flow was measured using a Validyne differential pressure transducer (Model DP-45161927; Validyne Engineering Corp., Northridge, CA) driven by a Gould carrier amplifier (Model 11-4113; Gould Electronic, Cleveland, OH). The esophageal balloon was attached to one side of the differential pressure transducer, and the outflow of the intratracheal tube was connected to the opposite side of the pressure transducer to allow recording of transpulmonary pressure. Flow was integrated to give a continuous tidal volume, and measurements of total lung resistance (RL) and dynamic compliance (Cdyn) were calculated at isovolumetric and flow zero points, respectively, using an automated respiratory analyzer (Model 6; Buxco, Sharon, CT).

Animals were randomized and on Day 1 pretreatment values for PC50 were obtained for aerosolized adenosine. Antisense (HAdA1AS) or mismatched control (HAdA1MM) oligonucleotides were dissolved in sterile physiological saline at a concentration of 5000 ug (5 mg) per 1.0 ml. Animals were subsequently administered the aerosolized antisense or mismatch oligonucleotide via the intratracheal tube (approximately 5000 ug in a volume of 1.0 ml), twice daily for two days.

Aerosols of either saline, adenosine, or antisense or mismatch oligonucleotides were generated by an ultrasonic

-18-

nebulizer (DeVilbiss, Somerset, PA), producing aerosol droplets 80% of which were smaller than 5  $\mu$ m in diameter.

In the first arm of the experiment, four randomly selected allergic rabbits were administered antisense oligonucleotide and four the mismatched control oligonucleotide. On the morning of the third day, PC50 values (the concentration of aerosolized adenosine in mg/ml required to reduce the dynamic compliance of the bronchial airway 50% from the baseline value) were obtained and compared to PC50 values obtained for these animals prior to exposure to oligonucleotide.

Following a 1 week interval, animals were crossed over, with those previously administered mismatch control oligonucleotide now administered antisense oligonucleotide, and those previously treated with antisense oligonucleotide now administered mismatch control oligonucleotide. Treatment methods and measurements were identical to those employed in the first arm of the experiment. It should be noted that in six of the eight animals treated with antisense oligonucleotide, adenosine-mediated bronchoconstriction could not be obtained up to the limit of solubility of adenosine, 20 mg/ml. For the purpose of calculation, PC50 values for these animals were set at 20 mg/ml. The values given therefore represent a minimum figure for antisense effectiveness. Actual effectiveness was higher. The results of this experiment are illustrated in both Figure 1 and Table 1.

TABLE 1. EFFECTS OF ADENOSINE  $A_1$  RECEPTOR ANTISENSE OLIGONUCLEOTIDE UPON PC50 VALUES IN ASTHMATIC RABBITS.

Mismatch Control		$A_1$ receptor Antisense oligonucleotide	
Pre oligonucleotide	Post oligonucleotide	Pre oligonucleotide	Post oligonucleotide
3.56 $\pm$ 1.02	5.16 $\pm$ 1.93	2.36 $\pm$ 0.68	>19.5 $\pm$ 0.34**

Results are presented as the mean (N = 8)  $\pm$  SEM. Significance was determined by repeated-measures analysis of variance (ANOVA), and Tukey's protected t test. \*\*Significantly different from all other groups, P < 0.01.

-19-

In both arms of the experiment, animals receiving the antisense oligonucleotide showed an order of magnitude increase in the dose of aerosolized adenosine required to reduce dynamic compliance of the lung by 50%. No effect of the mismatched control oligonucleotide upon PC50 values was observed. No toxicity was observed in any animal receiving either antisense or control inhaled oligonucleotide.

These results show clearly that the lung has exceptional potential as a target for antisense oligonucleotide-based therapeutic intervention in lung disease. They further show, in a model system which closely resembles human asthma, that downregulation of the adenosine A<sub>1</sub> receptor largely eliminates adenosine-mediated bronchoconstriction in asthmatic airways. Bronchial hyperresponsiveness in the allergic rabbit model of human asthma is an excellent endpoint for antisense intervention since the tissues involved in this response lie near to the point of contact with aerosolized oligonucleotides, and the model closely simulates an important human disease.

#### EXAMPLE 4

##### Specificity of A<sub>1</sub>-adenosine receptor

##### Antisense oligonucleotide

At the conclusion of the crossover experiment of Example 3, airway smooth muscle from all rabbits was quantitatively analyzed for adenosine A<sub>1</sub> receptor number. As a control for the specificity of the antisense oligonucleotide, adenosine A<sub>2</sub> receptors, which should not have been affected, were also quantified.

Airway smooth muscle tissue was dissected from each rabbit and a membrane fraction prepared according to described methods (J. Kleinstein and H. Glossmann, *Naunyn-Schmiedeberg's Arch. Pharmacol.* 305, 191-200 (1978), with slight modifications. Crude plasma membrane preparations were stored at - 70°C until the time of

-20-

assay. Protein content was determined by the method of Bradford (M. Bradford, *Anal. Biochem.* 72, 240-254 (1976)). Frozen plasma membranes were thawed at room temperature and were incubated with 0.2 U/ml adenosine deaminase for 30 minutes at 37°C to remove endogenous adenosine. The binding of [<sup>3</sup>H]DPCPX (A<sub>1</sub> receptor-specific) or [<sup>3</sup>H]CGS-21680 (A<sub>2</sub> receptor-specific) was measured as previously described. S. Ali et al., *J. Pharmacol. Exp. Ther.* 268, 1328-1334 (1994); S. Ali et al., *Am. J. Physiol* 266, L271-277 (1994).

As illustrated in both **Figure 2** and **Table 2**, animals treated with adenosine A<sub>1</sub> antisense oligonucleotide in the crossover experiment had a nearly 75% decrease in A<sub>1</sub> receptor number compared to controls, as assayed by specific binding of the A<sub>1</sub>-specific antagonist DPCPX. There was no change in adenosine A<sub>2</sub> receptor number, as assayed by specific binding of the A<sub>2</sub> receptor-specific agonist 2-[p-(2-carboxyethyl)-phenethylamino]-5'-(N-ethylcarboxamido) adenosine (CGS-21680).

**TABLE 2. SPECIFICITY OF ACTION OF ADENOSINE A<sub>1</sub> RECEPTOR ANTISENSE OLIGONUCLEOTIDE.**

	Mismatch Control oligonucleotide	A <sub>1</sub> Antisense oligonucleotide
A <sub>1</sub> -Specific Binding	1105 ± 48**	293 ± 18
A <sub>2</sub> -Specific Binding	302 ± 22	442 ± 171

Results are presented as the mean (N = 8) ± SEM. Significance was determined by repeated-measures analysis of variance (ANOVA), and Tukey's protected t test. \*\*Significantly different from mismatch control, P < 0.01.

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

-21-

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Nyce, Jonathan W.

(ii) TITLE OF INVENTION: METHOD OF TREATMENT FOR ASTHMA

(iii) NUMBER OF SEQUENCES: 6

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## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

## (viii) ATTORNEY/AGENT INFORMATION:

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(C) REFERENCE/DOCKET NUMBER: 5128-29

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## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GATGGAGGGC GGCATGGCGG G

-22-

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTAGCAGGCG GGGATGGGGG C

21

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTTGTTGGGC ATCTTGCC

18

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTACTTGCGG ATCTAGGC

18



-23-

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTGGGCCTAG CTCTCGCC

18

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTCGGGGTAC CTGTGGGC

18

## THAT WHICH IS CLAIMED IS:

1. A method of reducing adenosine-mediated bronchoconstriction in a subject in need of such treatment, comprising administering an adenosine receptor antisense oligonucleotide to the lungs of the subject in  
5 an amount effective to reduce bronchoconstriction, said adenosine receptor selected from the group consisting of A<sub>1</sub> adenosine receptors and A<sub>3</sub> adenosine receptors.
2. A method according to claim 1 wherein said adenosine receptor is the A<sub>1</sub> adenosine receptor.
- 10 3. A method according to claim 1 wherein said adenosine receptor is the A<sub>3</sub> adenosine receptor.
4. A method according to claim 1 wherein said antisense oligonucleotide comprises nucleotides in which at least one phosphodiester linkage is replaced with a  
15 linkage selected from the group consisting of methylphosphonate linkages, phosphotriester linkages, phosphorothioate linkages, phosphorodithioate linkages, and phosphoramidate linkages.
5. A method according to claim 1 wherein said  
20 antisense oligonucleotide has the sequence given herein as SEQ ID NO:1.
6. A method according to claim 1 wherein said antisense oligonucleotide has the sequence given herein as SEQ ID NO:3.
- 25 7. A method according to claim 1 wherein said antisense oligonucleotide has the sequence given herein as SEQ ID NO:5.

-25-

8. A method according to claim 1 wherein said antisense oligonucleotide is delivered by administering an aerosol of respirable particles containing said antisense oligonucleotide to the lungs of said subject.

5 9. A method according to claim 8, wherein said particles are selected from the group consisting of solid particles and liquid particles.

10 10. A method according to claim 9, wherein said aerosol is comprised of particles having a particle size within the range of about 0.5 to 10 microns.

11. A method according to claim 8 wherein said particles are liposomes containing said antisense oligonucleotide.

12. A method according to claim 8 wherein said  
15 antisense oligonucleotide is administered in amount sufficient to achieve intracellular concentrations of said antisense oligonucleotide in said subject from about 0.1 to 10  $\mu$ M.

13. A method of treating asthma in a subject  
20 in need of such treatment, comprising administering an adenosine receptor antisense oligonucleotide to said subject in an amount effective to treat asthma, said adenosine receptor selected from the group consisting of  $A_1$  adenosine receptors and  $A_2$  adenosine receptors.

25 14. A method according to claim 13 wherein said adenosine receptor is the  $A_1$  adenosine receptor.

15. A method according to claim 13 wherein said adenosine receptor is the  $A_2$  adenosine receptor.

-26-

16. A method according to claim 13 wherein said antisense oligonucleotide comprises nucleotides in which at least one phosphodiester linkage is replaced with a linkage selected from the group consisting of  
5 methylphosphonate linkages, phosphotriester linkages, phosphorothioate linkages, phosphorodithioate linkages, and phosphoramidate linkages.

17. A method according to claim 13 wherein said antisense oligonucleotide has the sequence given  
10 herein as SEQ ID NO:1.

18. A method according to claim 13 wherein said antisense oligonucleotide has the sequence given herein as SEQ ID NO:3.

19. A method according to claim 13 wherein  
15 said antisense oligonucleotide has the sequence given herein as SEQ ID NO:5.

20. A method according to claim 13 wherein said antisense oligonucleotide is delivered by administering an aerosol of respirable particles  
20 containing said antisense oligonucleotide to the lungs of said subject.

21. A method according to claim 20, wherein said particles are selected from the group consisting of solid particles and liquid particles.

22. A method according to claim 21, wherein  
25 said aerosol is comprised of particles having a particle size within the range of about 0.5 to 10 microns.

23. A method according to claim 20 wherein  
30 said particles are liposomes containing said antisense oligonucleotide.

-27-

24. A method according to claim 13 wherein said antisense oligonucleotide is administered in amount sufficient to achieve intracellular concentrations of said antisense oligonucleotide in said subject from about  
5 0.1 to 10  $\mu$ M.

25. A pharmaceutical composition, comprising, together in a pharmaceutically acceptable carrier:  
an adenosine receptor antisense oligonucleotide; said adenosine receptor selected from  
10 the group consisting of A<sub>1</sub> adenosine receptors and A<sub>3</sub> adenosine receptors; in an amount effective to reduce adenosine-mediated bronchoconstriction.

26. A method according to claim 25 wherein said adenosine receptor is the A<sub>1</sub> adenosine receptor.

15 27. A method according to claim 25 wherein said adenosine receptor is the A<sub>3</sub> adenosine receptor.

28. A method according to claim 25 wherein said antisense oligonucleotide comprises nucleotides in which at least one phosphodiester linkage is replaced  
20 with a linkage selected from the group consisting of methylphosphonate linkages, phosphotriester linkages, phosphorothioate linkages, phosphorodithioate linkages, and phosphoramidate linkages.

29. A method according to claim 25 wherein  
25 said antisense oligonucleotide has the sequence given herein as SEQ ID NO:1.

30. A method according to claim 25 wherein said antisense oligonucleotide has the sequence given herein as SEQ ID NO:3.

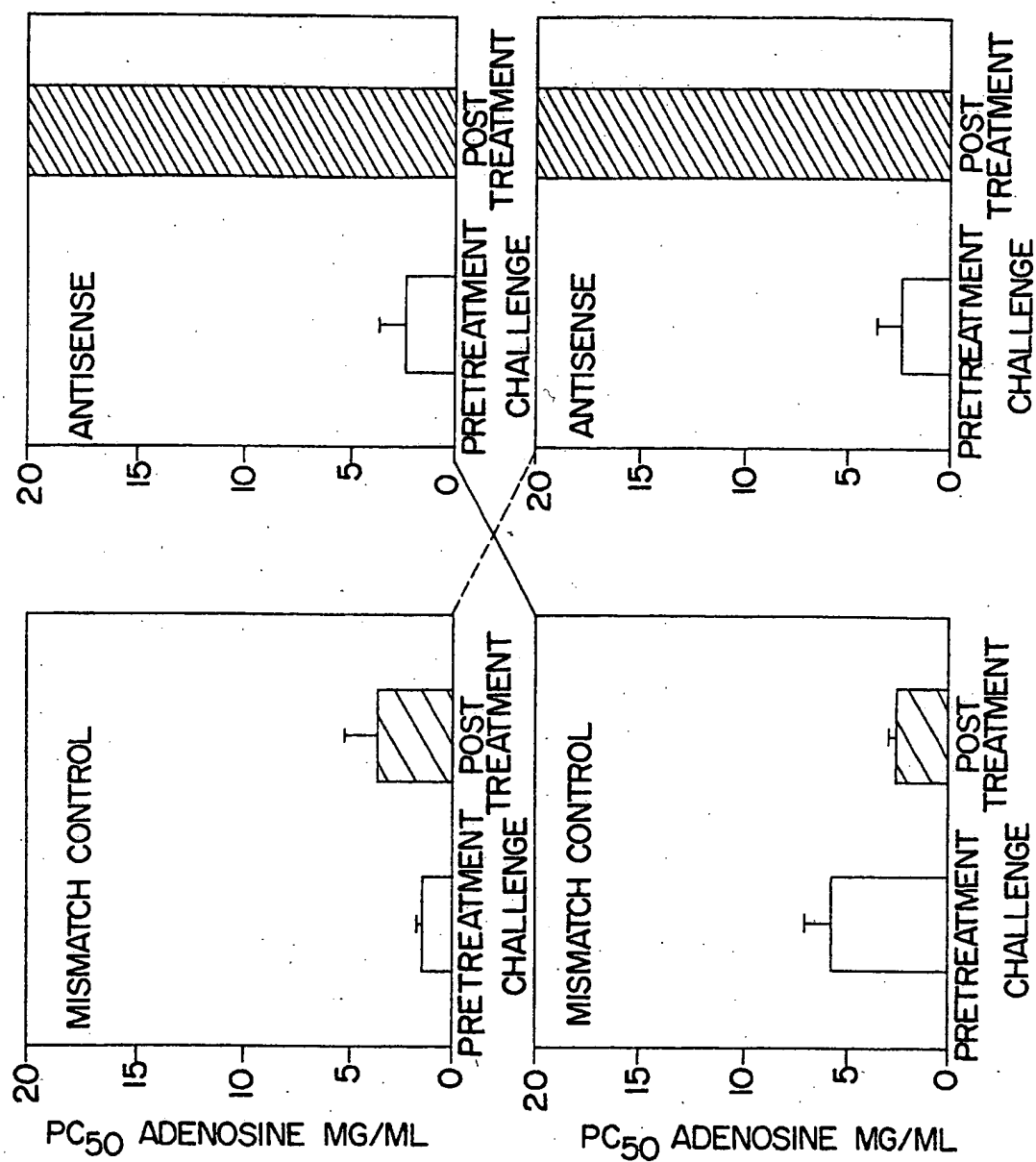
31. A method according to claim 25 wherein said antisense oligonucleotide has the sequence given herein as SEQ ID NO:5.

32. A pharmaceutical composition according to  
5 claim 25, wherein said carrier is selected from the group consisting of solid carriers and liquid carriers.

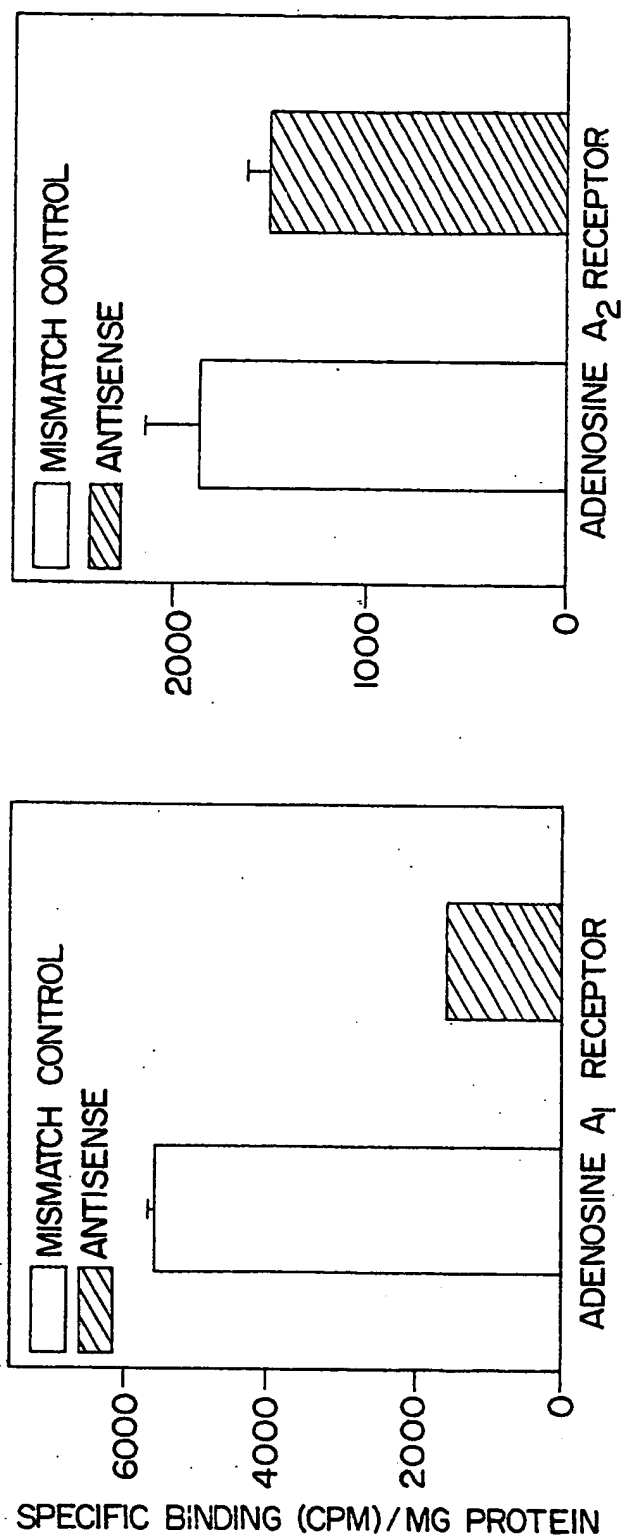
33. A pharmaceutical composition according to claim 25, further comprising a liposome, said liposome containing said antisense oligonucleotide.

10 34. A pharmaceutical composition according to claim 25, wherein said antisense oligonucleotide is conjugated to a molecule capable of cellular uptake.

1/2

FIG. 1.

2/2

FIG. 2.



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/08325

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 48/00; C07H 21/00

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, MEDLINE, CANCERLIT, EMBASE, CAPLUS, LIFESCI

search terms: adenosine, receptor, gene therapy, oligonucleotide, polynucleotide, antisense

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94/02605 A1 (DUKE UNIVERSITY) 03 February 1994 (03.02.94), whole document, specifically pages 4-5 and 20-21.	1-34
Y	ALI et al. Adenosine-induced bronchoconstriction in an allergic rabbit model: Antagonism by theophylline aerosol. Agents Actions. 1992, Vol. 37, pages 165-167, see entire document.	1-34
Y	ALI et al. Adenosine-Induced Bronchoconstriction and Contraction of Airway Smooth Muscle from Allergic Rabbits with Late-Phase Airway Obstruction: Evidence for an Inducible Adenosine A1 Receptor. The Journal of Pharmacology and Experimental Therapeutics. 1994, Vol. 268, pages 1328-1334, see entire document.	1-34

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

19 JULY 1996

Date of mailing of the international search report

29 JUL 1996

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/08325

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,225,326 A (J. BRESSER ET AL.) 06 July 1993 (06.07.93), whole patent, specifically columns 10-11.	3, 5-7, 15, 17-19, 27, 30, 31
Y	GB 2,264,948 A (MERCK & CO INC) 15 September 1993 (15.09.93), Figure 8.	3, 6, 7, 15, 18, 19, 27, 30, 31

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/08325

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/6, 69.1, 172.3, 91.1; 514/44; 935/62, 55, 56, 34, 54, 52, 70, 71, 65, 66; 536/24.5, 23.1, 23.4, 23.5

## B. FIELDS SEARCHED

Minimum documentation searched

Classification System: U.S.

435/6, 69.1, 172.3, 91.1; 514/44; 935/62, 55, 56, 34, 54, 52, 70, 71, 65, 66; 536/24.5, 23.1, 23.4, 23.5

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